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Bacterivory by benthic organisms in sediment: Quantification using ^{15}N-enriched bacteria

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Abstract

The fate of benthic bacterial biomass in benthic food webs is a topic of major importance but poorly described. This paper describes an alternative method for evaluation of bacterial grazing rate by meiofauna and macrofauna using bacteria pre-enriched with stable isotopes. Natural bacteria from the sediment of an intertidal mudflat were cultured in a liquid medium enriched with $^{15}NH_4Cl$. Cultured bacteria contained 2.9% of ^{15}N and were enriched sufficiently to be used as tracers during grazing experiments. Cultured bacteria presented a biovolume (0.21 μm^3) and a percentage of actively respiring bacteria (10%) similar to those found in natural communities. The number of Operational Taxon Units found in cultures fluctuated between 56 and 75% of that found in natural sediment. Despite this change in community composition, the bacterial consortium used for grazing experiments exhibited characteristics of size, activity and diversity more representative of the natural community than usually noticed in many other grazing studies. The bacterial ingestion rates of three different grazers were in the range of literature values resulting from other methods: 1149 ngC $ind^{-1}h^{-1}$ for the mud snail *Hydrobia ulvae*, 0.027 ngC ind^{-1} h^{-1} for the nematode community, and 0.067 ngC ind^{-1} h^{-1} for the foraminifera *Ammonia tepida*. The alternative method described in this paper overcomes some past limitations and it presents interesting advantages such as short time incubation and *in situ* potential utilisation.

Keywords: Bacteria; Grazing; Sediment; Stable isotope; Tracer

1. Introduction

Development of improved methods for measuring bacterial abundance and production has radically changed the perception of the role of bacteria in pelagic marine ecosystems. Bacteria are known to play a major role in organic matter degradation and regeneration of nutrients. Moreover the "microbial loop" model (e.g. Azam et al., 1983) considers bacteria as a "link" more than a "sink", increasing the ratio of primary production available for higher trophic levels. Therefore, bacteria appear to play a major role in pelagic food webs models (e.g. Vézina and Savenkoff, 1999).

Bacterial abundance in marine soft sediments is relatively constant, around 10^9 cells ml^{-1} porewater (Schmidt et al., 1998), being a thousand times more abundant than in pelagic systems. Moreover, high rates of production have been measured in aquatic sediments (e.g. van Duyl and Kop, 1990). These findings have driven a debate on the fate of bacteria in benthic food webs. Due to technical limitations, studies dealing with benthic bacterivory are not as developed as pelagic ones (Kemp, 1990).

Pelagic bacteria are mainly grazed by protozoa (e.g. Sherr and Sherr, 1994) and a similar pattern was expected in benthic systems (van Duyl and Kop, 1990; Bak et al., 1991; Hondeveld et al., 1994). Nevertheless, numerous authors consider the bacterial grazing by benthic ciliates and flagellates as insignificant (Alongi, 1986; Kemp, 1988; Epstein and Shiaris, 1992; Epstein, 1997). Depending on the studies, meiofauna grazing is considered either as (i) high enough to structure microbial communities (Montagna, 1984b), (ii) using 3% of bacterial production (van Oevelen et al., 2006a), or (iii) negligible (Epstein and Shiaris, 1992). Data on macrofaunal grazing rates are not less variable than on meiofaunal

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ones. In a synthesis, Kemp (1990) asserted that bacteria density is not high enough to play a major role in macrobenthos diet. These contrasting conclusions probably reflect the use of different methods. In conclusion, it appears that drawing a general view of the role played by microfauna, meiofauna and macrofauna in bacterial grazing is presently difficult (Kemp 1990).

Most of the benthic studies on trophic process employ tracers. Labels can be added directly to sediment. In such a situation, bacteria incorporate labels and are simultaneously grazed by predators (Montagna, 1995; van Oevelen et al., 2006a,b). This technique minimizes disturbance of the spatial distribution and metabolism of grazers and bacteria (Carman et al., 1989). Nevertheless, only a small part of the bacterial assemblages takes up detectable quantities of labels (Carman, 1990b). Moreover, the main drawback to this method is that a large part (up to 83% in Montagna and Bauer (1988)) of the total labels uptake may be attributable to processes other than grazing. Grazers may become labelled by absorption and adsorption of dissolved organic matter (DOM) (Montagna, 1984a) or by uptake of labels by non-prey microorganisms associated with grazers (e.g. epicuticular or gut microorganisms) (Carman, 1990a).

To reduce this bias, microbial prey can be pre-labelled with fluorescent products, or isotopes either stable or radioactive. Fluorescent Labelled Bacteria (FLB) with monodispersed FLB or whole-sediment staining methods are used mainly to assess grazing activity of small predators like flagellates and ciliates (Novitsky, 1990; Epstein and Shiaris, 1992; Hondeveld et al., 1992; Starink et al., 1994; Hamels et al., 2001). Meiofauna studies using FLB are seldom because FLB detection is difficult and time consuming in large sized grazers. Consequently, only a small number of specimens can be examined preventing detection of inter-individual variations in grazing rate. Therefore, grazers like nematodes are able to discharge various digestive enzyme to realise extracorporeal hydrolyse of food (Riemann and Helmke, 2002). In such a case, pre-digested FLB ingested are impossible to detect in grazers. Nevertheless bacterivory levels by foraminifera (Langezaal et al., 2005) and nematodes (Epstein, 1997) were assessed using FLB.

Bacterivory assessment by the way of adding radioactive or stable isotope to sediment was performed on meiofauna and macrofauna (e.g. Montagna, 1984b; Sundback et al., 1996; van Oevelen et al., 2006a,b).

Bacterivory assessment using pre-labelled bacteria was performed with radioactive isotopes (Rieper, 1978; Carman and Thistle, 1985). To our knowledge, the use of stable isotopes on pre-labelled bacteria has never been performed until present. Compared to radioactive isotopes, bacteria enriched with stable isotopes are more convenient to use, since they can be used *in situ* without negative environmental effects and legal restrictions. This method will help investigators who are limited by radioactive material prohibition.

The aim of this paper is to describe a method using ^{15}N stable isotope to pre-label bacteria in the view to assess bacterivory of large size benthic organisms (meiofauna and macrofauna). Experiments were performed mainly to assess the validity of this method, taking in consideration size, diversity, and activity of the pre-labelled bacteria in order to be close to natural population parameters. The method was applied to 3 grazers from an intertidal mudflat in order to appreciate its potential generalization: one mollusc *Hydrobia ulvae*, a nematode community and the foraminifera *Ammonia tepida*.

2. Material and methods

2.1. Bacterial culture

Superficial sediment (1 cm) was collected from the Brouage intertidal mudflat located in the eastern part of Marennes-Oléron Bay (45°55N, 1°06 W) on the Atlantic Coast of France. One ml of this sediment was added to 20 ml of bacterial liquid culture medium and kept in the dark at 13 °C during 24 h. The liquid bacterial culture medium was composed of: peptone 3 g l^{-1} (BioRad), yeast extract 1 g l^{-1} (BioRad), $^{15}NH_4Cl$ 1 g l^{-1} (99% ^{15}N-enriched NH_4Cl CortecNet); sodium glycerophosphate 0.025 g l^{-1} and sequestren Fe 6 g l^{-1}. It was completed with 0.2 μm filtered distilled water (500 ml) and 0.2 μm filtered sea water (500 ml) at pH 7.4. The first culture was subcultured during 24 h under the same incubation conditions in the view to reach approximately 2×10^9 cells ml^{-1}. Bacteria were rinsed (i.e. separated from culture medium) by the means of 3 centrifugations (3500 g, 10 min, 20 °C) in 0.2 μm filtered sea water, then frozen in liquid nitrogen and kept frozen (−80 °C) until grazing experiments.

The bacteria $\delta^{15}N$ was measured on an Eurovector Elemental Analyser coupled with an Isotope Mass Ratio Spectrometer (Isoprime, Micromass). Nitrogen isotope composition is expressed in the delta notation ($\delta^{15}N$) relative to air N_2: $\delta^{15}N = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{reference})^{-1}]\times1000$. Rinsing efficiencies were tested using bacteria cultured in the medium previously described with non-enriched NH_4Cl. These bacteria were killed by formalin (2%), placed in the ^{15}N-enriched culture medium previously described, harvested by the means of 3 centrifugations (3500 g, 10 min, 20 °C) before isotope ratio measurement.

2.2. Cultured bacteria size

Size of bacteria from original sediment and cultures were measured. For sediment samples, particle-associated bacteria were detached by pyrophosphate (0.01 M) and sonication. Bacteria were stained using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (2500 μg l^{-1}) and filtered onto 0.2 μm Nucleopore black filters (Porter and Feig, 1980).

Length (L) and width ($2r$) of each bacteria was determined by a computer-assisted image analysis (AxioVision Release 4.3) with an epifluorescence microscope (AxioSkop 2 mot plus-Zeiss) equipped with a charge-coupled device camera (AxioCam MRc5-Zeiss). Bacterial biovolumes (V) were calculated for cultured bacteria ($N=1981$) and natural bacteria ($N=1806$) as follows: $V=\pi r^2\cdot(L-2/3\ r)$ (Fuhrman, 1981).

2.3. Cultured bacteria activity

Frozen aliquots of cultured bacteria were thawed and immediately incubated with 5-cyano-2,3-ditoyl tetrazolium

chloride (CTC) (final concentration of 5 mM). After 2, 3 and 5 h of incubation, experiments were stopped with formalin (2%) and stored at 4 °C. Bacterial samples were processed as described above for the DAPI staining in order to simultaneously count total cells (UV excitation) and active cells (green excitation) on same slide.

2.4. Cultured bacteria diversity

The bacterial diversity of original sediment and culture aliquots was assessed. The DNA was extracted using an Ultraclean Soil DNA Kit (MO BIO, Oxyme) for sediment samples and a QIAamp DNA Mini Kit (Qiagen) for cultures. Bacterial 16S rRNA gene fragments of about 520 bp (the V6–V8 regions of 16r DNA (Gelsomino et al., 1999)) were amplified by PCR using primers 968fGC (5′-AACGCGAAGAACCTTAC-3′[with GC clamp 5′]) and 1401r (5′-CGGTGTGTACAAGGCCC-3′).

PCR products (300 ng) were loaded onto polyacrilamide gel (8% w/v, 7 M urea) in TAE 1 X buffer. Electrophoresis was processed under a constant voltage of 68 V, during 17 h, with a thermal gradient from 66 to 69.7 °C increasing at the rate of 0.2 °C h^{-1} (Dcode#System: BioRad). The gel was stained with 0.5 μg ml^{-1} Gelstar (BMA) in 1.25× TAE buffer during 30 min and checked through a UV transilluminator system (Versa Doc (Bio-Rad)) equipped with a camera. Temperature Gradient Gel Electrophoresis (TGGE) banding patterns were automatically calculated by the Bionumerix software (Applied Biomaths, Koutrai, Belgium) using the Dice coefficient (DC), without band weighing by both the complete linkage and unweighted pair group method with arithmetic mean (UPGMA) algorithms (threshold of 1%).

2.5. Grazing experiments

The first centimetre of sediment was collected from a square meter patch during ebb tide from the Brouage intertidal mudflat (France) on March 13, 2006. It was sieved on 500 μm, 200 μm and 50 μm in order to concentrate respectively *H. ulvae*, *A. tepida* and nematodes. Choice of these organisms was driven by their high natural abundance in the study area. Each type of grazer was placed in individual microcosms. Seventeen handpicked specimens of *H. ulvae* were placed in polypropylene Petri dishes (diameter 9 cm). For the foraminifera and nematode experiments, 1 ml of the fraction remaining on the 200 μm and on the 50 μm mesh sieves respectively were placed in 100 ml Pyrex beakers. Each experiment was carried out in triplicate, along with triplicate controls. Control samples were frozen (−80 °C) during 12 h in order to kill grazers before thawing.

Sediment that passed through the 50 μm mesh was mixed with ^{15}N-enriched bacteria. Abundance of sediment and cultured bacteria were counted using the methods previously described. This slurry containing 1.05×10^{9} bacteria ml^{-1} and ^{15}N-enriched bacteria were twice as abundant as natural ones. Seventeen ml of this slurry was placed in *H. ulvae* microcosms and 4 ml was placed in nematode and foraminifera microcosms. Grazing incubations were run in the dark at 20 °C. Incubations were stopped by freezing the microcosms at −80 °C.

H. ulvae were separated by hand from their shell and all specimens of each microcosm were pooled and homogenised using a Potter–Eveljhem. Nematodes were extracted from sediment using ludox (Heip et al., 1985). Approximately 700 nematodes were randomly handpicked from each sample. Foraminifera were stained with rose Bengal in order to identify living specimens. As Rose Bengal is an organic compound, it could affect isotopic composition but control experiments were also stained in order to take this bias into account. For each sample, 150 specimens of *A. tepida* were picked individually and cleaned of any adhering particles.

δ^{15}N of grazers was determined using the technique described above. Incorporation of ^{15}N is defined as excess (above background) ^{15}N and is expressed in terms of specific uptake (I) (gN ind^{-1}). I was calculated as the product of excess ^{15}N (E) and biomass of N per grazer. E is the difference between the fraction ^{15}N in the background ($F_{background}$) and in the sample (F_{sample}): $E=F_{sample}-F_{background}$, where $F=^{15}N/(^{15}N+^{14}N)=R/(R+2)$ and R=the nitrogen isotope ratio. For the $F_{background}$, we used control values measured with killed grazers (frozen). R was derived from the measured δ^{15}N values as: $R=((\delta^{15}N/1000)+1)\times R_{airN2}$ with $R_{airN2}=7.35293\times10^{-3}$ (Mariotti, 1982). The uptake of bacteria (gC $ind^{-1}h^{-1}$) was calculated as Uptake=($I\times$(% $C_{enriched\ bacteria}$/% $N_{enriched\ bacteria}$))/($F_{enriched\ bacteria}\times$incubation time). This uptake was multiplied by the ratio between the abundance of total and enriched bacteria, determined from DAPI counts. Uptake ($gC_{bacteria}/h/gC_{grazer}$) was obtained by dividing uptake of bacteria (gC/ind/h) by grazer mean weight (gC/ind).

3. Results

3.1. Characteristics of enriched bacteria

Bacteria cultured in a liquid medium with a 18 mM $^{15}NH_4Cl$ subsequently centrifuged to remove unincorporated label were found to contain 2.88±0.03% ^{15}N. Bacteria killed by formaldehyde before being placed in the same culture medium and centrifuged, contained 0.028% ^{15}N. Cultured bacteria were enriched enough to allow their detection in the three studied grazers (Table 1). Bacterial abundance was not affected by the liquid nitrogen freezing process.

Table 1
Calculation of ingestions rates of three different grazers

	Enriched bacteria	Gastropoda *H. ulvae*	Nematode community	Foraminifera *A. tepida*
% C by dry weight	35.2	33.6	38.2	5.8
% N by dry weight	10.2	8.0	7.4	0.8
Weight (g/ind)		5.4E-04	3.0E-07	1.8E-05
δ^{15}N living grazers	7068.2	95.0	20.6	20.0
δ^{15}N dead grazers		10.7	11.1	16.7
Incubation time (h)		2	5	5
Ratio (enriched/non-enriched bacteria) = 1.5				
Ingestion rate (ngC/ind/h)		1149.16	0.03	0.07
Ingestion rate (10^{-3} $gC_{bacteria}/gC_{grazer}/h$)		6.43	0.23	0.06

Cell volume of cultured bacteria (0.21 $\mu m^3 \pm 0.26$) was not significantly (bilateral unpairwise Student test; $p=0.07$) different from cell volume of natural bacteria (0.23 $\mu m^3 \pm 0.62$).

The ratio between active and non-active bacteria increased significantly with time in cultured bacteria (Analyse of variance, $p<0.01$) and evolved from 9.9 to 12.9% during the first 5 h after thawing.

The number of Operational Taxon Units (OTU) found in cultures fluctuated between 56 and 75% of that found in natural sediment (Fig. 2). The resulting dendrogram of TGGE patterns for cultured and natural sediment samples displayed two clusters. These clusters, of similar community composition, were defined by 49% pattern similarity. Subculturing does not seem to affect community composition to a great extent (75% of similarity) and did not change bacterial diversity. Freezing process induced a decline of 25% in the diversity of bacteria and slightly affected the bacterial community composition (84% of similarity).

3.2. Bacterial ingestion rates

After grazing experiments with pre-labelled bacteria, frozen grazers (control) were systematically less ^{15}N enriched than living ones for the three grazer types under study (Table 1). ^{15}N concentration increased linearly in grazers according to incubation time (Fig. 3). This linearity pointed that ingestion rates were constant during incubation period studied: 2 h for *H. ulvae* and 5 h for the nematode community and *A. tepida*. Raw data used for ingestion rates calculations are presented in Table 1. The mud snail *H. ulvae* grazed 1149 (± 0.285) ngC $ind^{-1}h^{-1}$, each nematode in the community grazed 0.027 (± 0.005) ngC ind^{-1} h^{-1} and the foraminifera *A. tepida* grazed 0.067 (± 0.013) ngC ind^{-1} h^{-1}.

4. Discussion

4.1. Discussion on methodology

4.1.1. Success of enrichment

Rinsing efficiency was tested by placing non-enriched killed bacteria in enriched medium and by separating them from this medium by centrifugations. Those bacteria were poorly enriched in ^{15}N, showing that the bacterial rinsing centrifugation process was efficient. Thus, ^{15}N enrichment of bacteria was due to a bacterial assimilation and not to culture medium remaining between bacterial cells. This high bacterial rinsing efficiency is essential, since some grazers are able to consume directly DOM (Montagna and Bauer, 1988) from the culture medium.

There is one disadvantage in using ^{15}N instead of ^{13}C enriched bacteria. As grazers contain more C than N, more biomass is required for isotopic measurements. However, the use of ^{15}N avoids the decalcification step required by ^{13}C and bias associated with this decalcification (Jacob et al., 2005). Moreover, grazing experiences are based on the assumption that isotopic composition of bacteria remains constant during the incubation period. The isotopic composition of ^{13}C enriched bacteria will vary quickly due principally to respiration loss and to a lower degree to production of DOM (Ogawa et al., 2001; Kawasaki and Benner, 2006). The use of ^{15}N permits to limit this respiration loss bias so isotopic composition of bacteria remains more stable during incubation.

4.1.2. Size of cultured bacteria

Discrimination of prey by grazers on the basis of size can influence the estimate of total bacterivory. Bacterial selection according to size has been well documented in planktonic protozoa (Pérez-Uz, 1996; Hahn and Höfle, 1999). Most protists graze preferentially on medium-sized bacterial cells, grazing being less efficient with smaller and larger cells (review in Hahn and Höfle, 2001). The soil nematode, *Caenorhabditis elegans* feeds on bacteria suspended in liquid and smaller bacteria are better food sources than larger ones for this species (Avery and Shtonda, 2003). Since, in our study, cultured and natural bacteria presented a similar average size, it can be inferred that there is only a small bias if any due to cell size selection by grazers.

4.1.3. Activity of cultured bacteria

Few data are available on CTC activities of natural benthic bacteria for comparison with our results. In superficial sediments from intertidal mudflats, 4 to 20% of benthic bacteria were found to be active (van Duyl et al., 1999). Proctor and Souza (2001) found 9 to 10% active cells in river sediments and 25% in intertidal sediments in the Gulf of Mexico. Haglund et al. (2002) detected 46% active bacteria in lake sediments.

Enriched bacteria with activity levels different from those of the natural community may induce a bias if grazers select bacteria according to prey activity. Nematodes can discriminate bacteria exhibiting different physiological or nutritional states (Grewal and Wright, 1992). *Pellioditis marina* do not assimilate heat-killed bacteria even though it feeds on live cells at high rates (Moens, 1999). In contrast, *Diplolaimelloides meyli* is more attracted by killed than by live bacteria (Moens et al., 1999a). The foraminifera *Ammonia beccarii* collects dead and living stained bacteria without discrimination (Langezaal et al., 2005).

Many existing methods to quantify bacterivory use bacterial communities with activity levels different from those of the natural communities. For instance, labels directly added to the sediment are only incorporated by the active fraction of the bacterial community: 100% of labelled bacteria are active. In contrast, pre-labelled bacteria such as standard FLB are generally dead (heat-killed): 0% of labelled bacteria are active. With the method described in our study, 10% of labelled bacteria are active after thawing of frozen cultures (Fig. 1). This activity is included in range found in natural environments (van Duyl et al., 1999; Proctor and Souza, 2001; Haglund et al., 2002). With the present method, grazers have the opportunity to pick up active or inactive bacteria according to their preference like in the natural environment.

4.1.4. Diversity in the cultured bacteria

Subculturing of bacteria produces a final culture free of sediment. Freezing allows storage of aliquots that may be

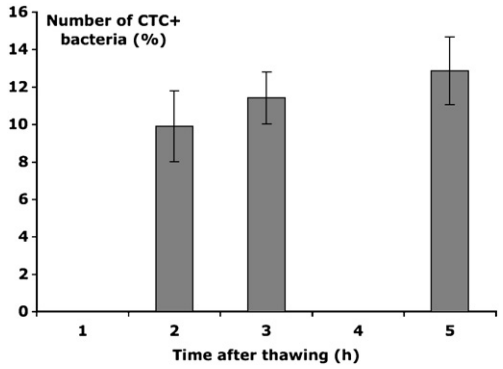

Fig. 1. Evolution of the percentage of CTC+cells related to total bacteria after thawing cultured bacteria. Bars indicate standard deviation ($n=3$).

enriched under standardised conditions at any time. The freezing step induces small variations in the diversity of the bacterial community that must be nevertheless kept in mind when using this method.

Grazers may be highly selective of prey species. To our knowledge, selection of bacteria has never been observed for macrofauna but demonstrated for nematodes (Moens et al., 1999a) and foraminifera (Lee et al., 1966; Bernhard and Bowser, 1992; Langezaal et al., 2005).

Nematodes used to be considered as generalist feeders, but they were recently shown to be selective feeders exhibiting various preferences for algal and microbial prey. Their reproduction rates differ according to the ingested strain of bacteria (Venette and Ferris, 1998; Blanc et al., 2006). Moens et al. (1999a) show that monhysterid nematodes are able to select bacterial strain. Selection can be due to the bacterial size: filamentous bacteria escape uptake by nematodes with small buccal cavities (Blanc et al., 2006). Moens et al. (1999a) consider that the chemotaxic responses of nematodes to their bacterial prey may be due more to chemical cues produced by the bacteria than to bacterial cell-wall structure that determine their palatability. Nematodes are also able to significantly modify the composition of a bacterial community by their species-specific bacterial food preferences (De Mesel et al., 2004).

The foraminifera *A. beccarii* distinguishes food and non-food particles during collection (Langezaal et al., 2005). Two allogromiidae species (Foraminifera) have been shown to be non-selective grazers, actively harvesting bacterial biofilm from 3 different inocula (Bernhard and Bowser, 1992). Lee et al. (1966) found that most species of bacteria do not serve as food for foraminifera whereas selected species of bacteria are consumed in large quantity.

Each bacterial species presents characteristics such as cell surface, nutritional quality or chemical cues which may influence bacterial grazer behaviour. These differences have not been evaluated between cultured versus natural bacteria in the present study. However, estimation of total community composition and diversity gives us an approximate idea of these differences. This molecular approach has the advantage to target dominant community members. The cultured community presents 49% of similarity with the natural bacterial community. Although cultivation of natural bacteria induces a shift in community composition (Fig. 2), this bacterial consortium seems more representative of the natural community than that of many other grazing studies. The majority of experiments that use FLB are done with monospecific bacteria or with a really limited number of bacterial species. Even if natural and cultured community are not strictly identical, the probability for grazers to find and ingest their preferred bacterial species is higher in the supplied bacterial consortium than with monospecific bacteria.

4.1.5. Characteristics of grazing experiment

All various methods developed and applied to measure bacterivory in natural communities posses methodological shortcomings that make interpretation of the resulting data problematic. The method presented in this study, using ^{15}N pre-enriched bacteria also presents bias. Sieving the sediment changes the bacterial availability for predators, bacteria being not attached to particle as in natural situation. The best way to minimize this artefact is to add the label directly to sediment in order to label bacteria while they are being grazed. This method is problematic as a high fraction of label found in grazers is due to processes other than grazing as underlined before. This requires control of incubations with a prokaryote activity inhibitor and the effectiveness of this inhibitor has to be tested for each grazer. The pre-enriched bacteria technique does not require the use of such inhibitors and only necessitate one control to determine adsorption of enriched bacteria on grazers.

Nematodes (Gerlach, 1978), foraminifera from the genera *Ammonia* (Chandler, 1989) and *H. ulvae* secrete mucus. During experiments with pre-enriched bacteria, controls must be performed to determine abundance of enriched bacteria stuck in the mucus secreted by grazers. Stuck bacteria modify the isotopic composition of grazers and controls are required to evaluate this bias due to non-grazing processes. In this study,

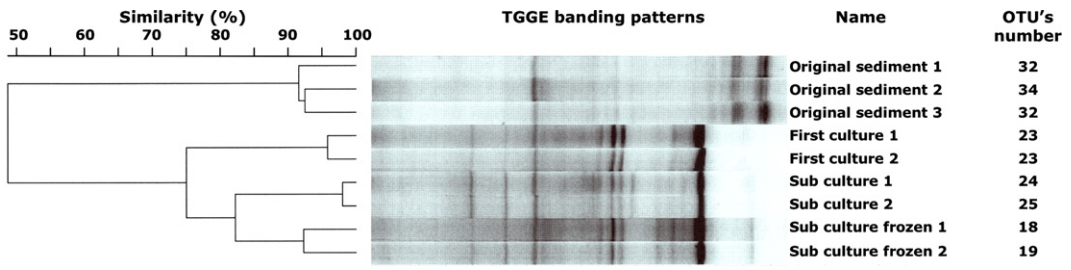

Fig. 2. TGGE analyses of natural sediment, first culture, subculture from the first culture and subculture from the first frozen culture. The right panel shows the relating band similarity (%) of bacterial communities.

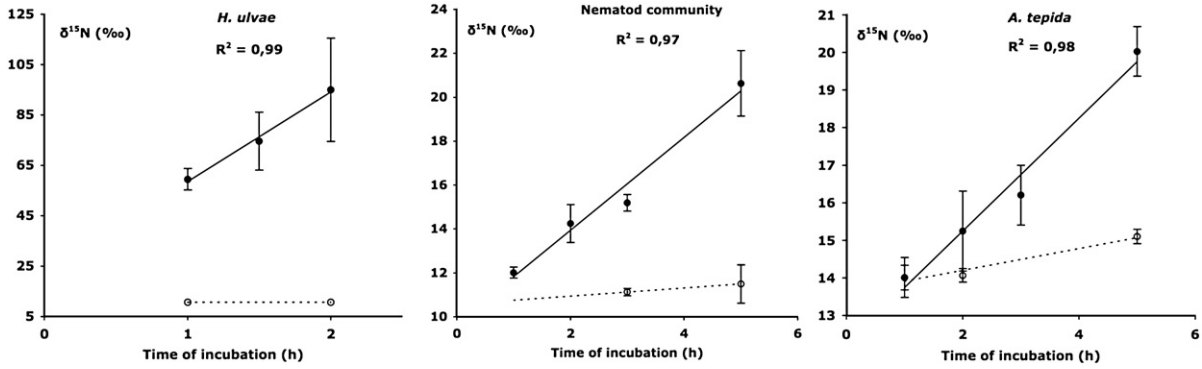

Fig. 3. Evolution of isotopic composition of three types of living (●) and dead (○) grazers placed in contact with ^{15}N-enriched bacteria. Bars indicate standard deviation (n=3).

freeze-killed grazer controls were used to determine this adsorption assuming that mucus post-freezing and mucus never frozen absorb bacteria at the same rate.

During grazing experiments, prey egestion from grazers may occur when chemical preservatives are used and this leads to underestimating grazing rates. Nematodes can egest a significant part of their gut contents when killed with formaldehyde (Moens et al., 1999b). In this study, grazers were frozen at −80 °C to reduce this bias.

Prior to grazing experiments, enriched bacteria were frozen in liquid nitrogen to prevent spontaneous and enzymatic degradation and maintain their viability. Bacteria are commonly cryopreserved during long-term storage in liquid nitrogen. Some agents like glycerol and methanol can be used to enhance cryopreservation, but in the case of grazing experiments, they can be toxic to grazers. Low-temperature storage enables standardised cultures, helping to ensure reproducible results in a series of experiments. This storage is really useful when monitoring over long periods is considered.

The method used allows short incubations that limit bias due to recycling. Bacterial ingestion is detectable after 2 h of incubation for 3 grazers (Fig. 3). During incubation, labelled bacteria may be first ingested by grazers that are themselves preyed by studied grazers. In such a situation, it is impossible to determine the part of label present in studied grazers that is provided respectively by bacteria and first grazers. Even if a short incubation time does not prevent this type of bias, it reduces it substantially.

4.2. Demonstration of applicability

Data from literature to compare with our values are scarce. First, these predator species have not been systematically studied. Secondly, herbivory is more commonly studied than bacterivory. Thirdly, the manner in which to report grazing rates depends on the aim of the study. Studies dealing with carbon flow generally report values on a biomass basis (e.g. ngC ind^{-1} h^{-1}). When the aim of the study is the impact of grazers on microbial community,

Table 2
Ingestion rate of bacteria observed in this study and compared with data from the literature concerning bacterivory and herbivory

Grazers	Grazing rate (10^{-3} $gC_{bacteria}$ gC_{grazer}^{-1} h^{-1})	Grazing rate ngC ind^{-1} h^{-1})	Labelling method	References
Gastropoda				
Hydrobia ulvae		1149.16	Stable isotope pre-enriched bacteria	Present study
Hydrobia ulvae	6.43	40–2080	Radioactive pre-labelled algae	(Haubois et al., 2005)
Hydrobia ulvae		896–1064	Radioactive pre-labelled algae	(Blanchard et al., 2000)
Hydrobia truncata		506–2873	Radioactive pre-labelled algae	(Forbes and Lopez, 1989)
Nematode				
Mudflat Nematode community	0.23	0.03	Stable isotope pre-enriched bacteria	Present study
Mudflat Nematode community	2.59–3.66		*In situ* radioactive labelled bacteria	(Montagna, 1984b)
Subtidal Nematode community	0.01		*In situ* radioactive labelled bacteria	(Montagna et al., 1995)
Plectrus palustris		10.54[a]	Radioactive pre-labelled bacteria	(Duncan et al., 1974)
Monhystera disjuncta		0.15–0.49[a]	Radioactive pre-labelled bacteria	(Herman and Vranken, 1988)
Diplolaimelloides meeyli		11–17	Radioactive pre-labelled bacteria	(Moens and Vincx, 2000)
Pellioditis marina		55–60	Radioactive pre-labelled bacteria	(Moens and Vincx, 2000)
Mudflat Nematode community		0.02[a]	Fluorescent pre-labelled bacteria	(Epstein and Shiaris, 1992)
Foraminifera				
Ammonia tepida	0.06	0.07	Stable isotope pre-enriched bacteria	Present study
Ammonia tepida	2.18	2.18	Stable isotope pre-enriched algae	(Moodley et al., 2000)
Ammonia beccarii		5×10^{-4}–1.6×10^{7}[a]	Fluorescent pre-labelled bacteria	(Langezaal et al., 2005)

[a] Ingestion rate converted with a bacterial biomass of 35 fgC cell^{-1} (Theil-Nielsen and Søndergaard, 1998).

grazing rates are generally reported as rate constants (e.g. h^{-1}) (Montagna, 1995).

While meiofaunal grazers are adapted to pick out specific microbial particles, macrofaunal deposit feeders process large volumes of sediment. Mud snails of the genus *Hydrobia* assimilate epipelic diatoms and attached bacteria (Newell, 1965; Kofoed, 1975; Lopez and Levinton, 1978; Jensen and Siegismund, 1980) contained in the ingested sediment. To our knowledge bacterial ingestion rates have never been determined but data is available concerning algal ingestion rates. The bacterial ingestion rate found in our study is in the same range as algal ingestion rates found by Forbes and Lopez (1989), Blanchard et al. (2000) and Haubois et al. (2005) (Table 2).

In literature, grazing rates of nematodes are strongly variable with a range of fluctuations of more than two orders of magnitude (Table 2). Thus, comparison of our data with literature is difficult. Those discrepancies may arise from a lot of reasons such as the use of different techniques or the experimental conditions. When grazing experiments are performed in monoxenical conditions, nematodes are in an environment constituted by water (or agar) and bacteria. Nematodes would probably present higher grazing rates in such conditions than during grazing experiments where bacterial food is mixed with minerals and refractory organic matter and therefore is less available. However, when our results are compared to values resulting from experiments using nematodes from mudflat grazing on labelled bacteria mixed with sediment, it appeared that they are in the range of grazing rates found by Epstein and Shiaris (1992) but more than ten times lower than those found by Montagna (1984b).

Algal ingestion rates by *A. tepida* are higher (Moodley et al., 2000) than bacterial ingestion rates found in our study (Table 2). Langezaal et al. (2005) used FLB in simplified microcosms with one specimen of *A. beccarii* in a reduced volume of water. Their bacterial grazing rate is lower than ours. This may be linked to the bacterial concentration used in microcosms (1.4×10^3 cell ml^{-1}), which is considerably lower than benthic bacterial abundance in the natural environment (c.a. 10^9 cell ml^{-1}) and in the present study.

5. Conclusion

The fate of benthic bacterial biomass is a topic of major importance in microbial ecology and in food web studies. All various methods developed and applied to measure bacterivory in natural communities possess artefacts and difficulties that make interpretation of the resulting data problematic. Our experimental approach is not an exception and also presents shortcomings. These bias are due principally to sediment manipulation. Labelled bacteria are not available to bacterivores in the same manner as bacteria in unmanipulated sediment.

However, grazing experiments with ^{15}N pre-enriched bacteria also present several advantages: (i) they can be performed *in situ* without environmental consequences, (ii) they do not require long incubations, so bias due to recycling is minimized, (iii) they require quite simple control tests with freezing of enriched prey, (iv) they can be performed at different times under standardised conditions, (v) they can be extended to other types of sediment or soil and (vi) they can be used in double-labelling experiments with ^{13}C enriched algae, in order to simultaneously measure bacterial and algal ingestion rates.

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